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Determination of Selected Pyrrolizidine Alkaloids in Honey by Dispersive Liquid−Liquid Microextraction and Ultrahigh-Performance Liquid Chromatography−Tandem Mass Spectrometry

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ABSTRACT: The contamination of honey with hepatotoxic pyrrolizidine alkaloids (PAs) is an actual concern for food safety.
This study reports the first application of dispersive liquid−liquid microextraction (DLLME) in the determination of five
relevant PAs, and the relative N-oxide derivatives (PANOs), in honey. The effects of different experimental parameters (pH,
ionic strength, type and volume of DLLME solvents) affecting the extraction efficiency were carefully investigated and optimized. PAs were extracted from honey (diluted solution 10% w/v at pH 9.5) by injecting a mixture of chloroform and isopropyl alcohol. A reduction step (zinc powder in acidic aqueous solution) before DLLME was performed to convert PANOs in PAs and to obtain the total PA levels. Both sample preparation protocols (DLLME and Zn-DLLME) showed negligible matrix effects on PA signal intensity in honeys of different botanical origins. The overall recoveries of DLLME and Zn-DLLME ranged from 71 to 102% and from 63 to 103%, respectively, with a good precision (standard deviations in the range from 1 to 12%). The attained method quantification limits stayed between 0.03 and 0.06 μg kg−1, and the linear response range extended to 25 μg kg−1. Additionally, the proposed method provides results comparable to those of the SPE protocol in the analysis of real samples. An analysis of retail honeys revealed PA residues in all analyzed samples, with a maximum level of 17.5 μg kg−1 (total PAs). Globally, the proposed method provides a sensitive and accurate determination of analytes and offers numerous advantages, such as simplicity, low cost, and a high sample throughput, which make it suitable for screening and quality control programs in food chain and occurrence studies.

KEYWORDS: pyrrolizidine alkaloids, dispersive liquid−liquid microextraction, honey, zinc reduction

■ INTRODUCTION

Pyrrolizidine alkaloids (PAs) are secondary metabolites produced by a wide array of plants as a defense mechanism against insect herbivores. The main sources (about 95% of known PA) are Boraginaceae (all genera), Asteraceae (tribes Senecionae and Eupatoriae), and Fabaceae (genus Crotalaria) families.1 PAs are tertiary amines with the structure of the necine base. Depending on the esterification of the two hydroxyl groups with necic acids, PAs occur as monoesters, open-chain diesters, and cyclic diesters.2,3 In addition, these alkaloids are naturally occurring as N-oxide derivatives (PANOs), which often coexist with PAs in plant materials.1−3 PAs are probably the most widely distributed natural toxins and therefore affect wildlife, livestock, and humans.1 Especially, 1,2-unsaturated PAs are considered highly hepatotoxic and genotoxic carcinogens, as they undergo metabolic activation to reactive pyrrole species, which can readily react with protein and form DNA adducts.1,4 Acute poisoning with 1,2-unsaturated PAs in humans is associated with high mortality and is characterized by hepatic veno-occlusive disease, whereas a subacute or chronic onset may lead to liver cirrhosis and pulmonary arterial hypertension.1,4 The available information indicate that a genotoxic and carcinogenic mechanism is applicable for all classes of 1,2-unsaturated PAs and their

Noxides, which can be metabolically converted into PAs.1,5 PAs can unintentionally enter the food chain when food products are either contaminated with or derived from PA containing plant tissues. Human poisoning was reported following treatment with herbal medicines and infusions of PA-containing plants and the intake of grain crops contaminated with PAs.4 Recent occurrence data revealed a chronic dietary exposure to PAs via the consumption of plant-derived foods contaminated by relatively low but significant PA levels,3,6,7 highlighting that PAs in foods is a relevant food safety issue.8 Thus, PAs are regarded as undesirable substances in food and feed and have been the subject of recent European Food Safety Authority (EFSA) opinions.1,5,9 On the basis of occurrence and toxicological data, the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) concluded that there is a possible concern for human health related to the exposure to PAs, in particular for frequent and high consumers of honey,1 tea, herbal infusions, and food supplements.5,9 Consequently, the CONTAM Panel proposed a list of the most relevant PAs to be monitored in food products, considering both their contribution to the total levels and their possible toxicological potencies.5 EFSA recommended continuing to collect occurrence data on PAs and PANOs in relevant food products and underlined the urgent need to develop analytical methods to determine individual and total PAs in food matrices and to decrease the uncertainties in the exposure ssessment.9

Honey is one of the most studied food matrices with respect to PA contamination. It contains significant PA levels that could cause chronic diseases if regularly consumed at recommended serving sizes (15−25 g) or at higher consumption levels.5,6,10 PAs found in honey may have been introduced by nectar-collecting bees, and PA types and levels depend on plants distributed in the collection area. In European honeys, lycopsamine-type PAs, in particular lycopsamine and echimidine, were the most important PAs in terms of contribution to the levels of PAs (81%).5 Lycopsamine-type PAs were also determined as dominant PAs in the contaminated Chinese (lycopsamine and echimidine) and Ghanaian (lycopsamine stereoisomers and corresponding Noxides) honeys.11,12 Moreover, clivorine was found with a high prevalence of PA contamination in honeys sold in China.11 Differently, senecionine-type PAs (senecionine N-oxide and senecionine) quantitatively predominated in positive Brazilian honey samples, while the lowest concentration and prevalence of echimidine was observed.13

In the last year several analytical methods for PA analysis have been developed. LC-MS techniques have emerged as the most versatile and useful detection method, because they allow a more sensitive and selective determination of PAs and PANOs, attaining very low detection limits, and provide much more reliable data with much less sample preparation.2 With regard to the sample preparation, at present solid-phase extraction (SPE), with C18 or strong cation exchange sorbents, has become a frequently used procedure for the simultaneous analysis of PAs and PANOs in food matrices.1,2,7 Although SPE allows an effective isolation of analytes, it is time consuming, relatively expensive, and needs to be tested and adapted to the different matrices.14,15 Notably, sample preparation recently is requiring attention by emphasizing simplification, miniaturization, and automation of the extraction procedure in order to make this analytical step “green”. In this regard, one of the suggested analytical practices implies the elimination or reduction of the amounts of solvents and reagents used in the extraction and/or cleanup steps. Dispersive liquid−liquid microextraction (DLLME) is a powerful preconcentration technique that has received special attention because of its numerous advantages such as simplicity, low cost, short extraction times, high enrichment factors, and modest consumption of organic solvents, which reduces their environmental impact.16−18 DLLME has been used to determine a wide variety of organic and inorganic compounds in clinical, forensic, food, and environmental matrices, and it has displayed performance comparable or superior to conventional methods.17−19 In this study the application of DLLME coupled to UHPLCMS/MS was evaluated to develop an inexpensive multiresidue method for the determination of PAs in honey at ultratrace levels (ppt). Based on the chemical characteristics of PA classes, their distribution in food products, and the possible toxicological potencies, five PAs (intermedine, Im; lycopsamine, Ly; senecionine, Sn; retrorsine, Re; echimidine, Em) and four PANOs (intermedine- -oxide, ImNO; senecionine Noxide, SnNO; retrorsine N-oxide, ReNO; echimidine N-oxide, EmNO) were selected as target analytes

(Figure S1 in the Supporting Information) among PAs of relevance in foods.5 The experimental parameters affecting DLLME efficiency were carefully studied and optimized using a multilevel experimental design. Zn reduction was employed to obtain an accurate quantification of total PA (PAs and PANOs) levels, due to the low DLLME extraction efficiency of PANOs. The analytical performance of the optimized procedure was determined, and the proposed procedure was compared with the method most widely used in the analysis of PAs in honey (strong cation exchange SPE).6 Finally, it was applied to the analysis of retail honeys. To the best of our knowledge, this report describes the first application of DLLME for the determination of PAs in food matrices.

■ MATERIALS AND METHODS

Reagents and Standards. Analytical-grade acetonitrile (MeCN), ethanol (EtOH), chloroform (CHCl3), dichloromethane (DCM), isopropyl alcohol (iPrOH), methanol (MeOH), tetrahydrofuran (THF), Zn powder (particle size <10 μm), and MS-grade formic acid (HCOOH) were obtained from Sigma-Aldrich (Milan, Italy). Ultrapure water (18 MΩ) was prepared by a Milli-Q purification system (Millipore, Bedford, MA, USA). MS-grade MeCN and water were supplied by Romil (Cambridge, U.K.). Reference standards (≥98% HPLC grade) of echimidine (Em), echimidine N-oxide (EmNO), intermedine (Im), intermedine Noxide (ImNO), lycopsamine (Ly), retrorsine (Re), retrorsine N-oxide (ReNO), senecionine (Sn), and senecionine N-oxide (SnNO) were purchased from PhytoLab GmbH & Co (Vestenbergsgreuth, Germany). Standard stock solutions (1 mg mL−1) of each compound were prepared in MeOH and stored at 4 °C. Diluted solutions and standard mixtures were prepared in MeOH/H2O 2/8 v/v. Stability tests of PA stock solutions (MeOH at 4 °C) and diluted standard mixtures (aqueous and sugar solutions at pH 9.6 for 24 h at ambient temperature) were carried out, and no degradation phenomena were observed.

Honey Samples. Honey samples (n = 25) were obtained from the Italian retail markets in the Campania area, in attempts to cover a wide range of product types, brands, and price levels. Collected samples comprised multifloral (n = 11) and monofloral honeys (3 acacia, 2 citrus, 2 sulla, 2 eucalyptus, 1 thyme, 1 lavender, 1 rosemary, 1 cherry and 1 strawberry honey), as indicated on the commercial label. The geographic origin of samples was mainly specified as “EC countries” (22 samples, of which 15 were Italian honeys) or blends from “EU and non-EU countries” (3 samples).

In the DLLME optimization studies, an artificial honey (supersaturated sugar solution at 80%, w/w containing 45 g of fructose and 35 g of glucose in 100 g of solution),20 spiked with target compounds at the 25 μg kg−1 level, was used as a test matrix.

UHPLC-MS/MS Analyses. Chromatographic analyses were performed using a Shimadzu Nexera X2 UHPLC system (Shimadzu, Milano, Italy) coupled to a Qtrap 6500 mass spectrometer (AB Sciex, Milan, Italy) equipped with a TurboV ion source. Analyst software (Version 1.6, Sciex) was used for instrument control, data acquisition, and data analysis.

A Luna Omega Polar C18 100 × 2.1 mm i.d., 1.6 μm column (Phenomenex, Bologna, Italy), with a corresponding precolumn, was used at a flow rate of 400 μL min−1 and at a temperature of 30 °C.

The mobile phase was a binary gradient of water (A) and MeCN (B), both containing 0.1% HCOOH. The gradient elution program is as follows: 0−1 min, 2% B; 1−5.5 min, 2−8% B; 5.5−7.5 min, 8% B; 7.5−9.5 min, 8−12% B; 9.5−11 min, 12−18% B; 11−13 min, 18−20% B. After each injection (5 μL), cleaning (98% B, 6 min) and reequilibration of the column (6 min) were performed.

The mass spectrometer was operated in positive ionization mode.

Nitrogen was used as the collision gas (CAD), and it was set as the “medium” mode. The MS and MS/MS parameters were optimized by infusing at a flow rate of 5 μL min−1 individual solutions of the analytes at 1 μg mL−1. The mobile phase and flow rate dependent source parameters were optimized under the chosen chromatographic conditions by injecting 10 ng mL−1 PA standard solutions. Optimized mass spectrometer parameters were as follows: curtain gas (CUR), ion source gases GS1 and GS2 35, 40, and 50 psi, respectively; ion spray voltage (IS) +5000 V; source temperature 400 °C; entrance potential (EP) +10 V; cell exit potential (CXP) +12 V; declustering potential (DP) +110 V. Three multiple reaction monitoring (MRM) transitions were monitored for

each PA, except for Em. MRM parameters were as follows: Em, m/z precursor/product transitions 398 → 120 (collision energy [CE] 31 V) and 398 → 220 (CE 25 V); EmNO, m/z 414 → 120 (CE 31), 414 → 137 (CE 43), and 414 → 254 (CE 40); Im and Ly, m/z 300 → 94 (CE 33), 300 → 120 (CE 32), and 300 → 156 (CE 39); ImNO, m/z 316 → 94 (CE 43), 316 → 138 (CE 36), and 316 → 172 (CE 38); Re, m/z 352 → 94 (CE 45), 352 → 120 (CE 40), and 352 → 138 (CE 40); ReNO, m/z 368 → 94 (CE 60), 368 → 118 (CE 40), and 368 → 136 (CE 45); Sn, m/z 336 → 94, 336 → 120, and 336 → 138 (CE 40); SnNO, m/z 352 → 94 (CE 45), 352 → 118 (CE 40), and 352 → 136 (CE 45).

Experimental Design. To optimize the best DLLME conditions, a chemometric approach (response surface) was applied, using the Statgraphic Centurion XVI software, version 16.1 (Statistical Graphics, Rockville, MD, USA). The study was performed on a 10% (w/v) aqueous solution of artificial honey fortified with 25 μg kg−1 of each PA. The pH of the aqueous solution was fixed at 9.6.

Box−Behnken design 2-factor interactions, with 21 degrees of freedom, 2 block replicates of 16 randomized experimental runs, and 4 center points for block, was applied. The experimental factors studied were as follows: A, salt amount (NaCl, range 15−25% w/v); B, extractant volume ($CHCl_3$, range 400−1000 μL); C, disperser volume (iPrOH, range 500−1000 μL). High and low levels of experimental factors were selected according to preliminary experiments.

Extraction efficiencies (EEs) of PAs and PANOs were considered as response variables. The experimental conditions of design and the values for the response variables are given in Table S1 of the Supporting Information. The statistical significance of the experimental factor contributions, and their first-order interactions, was determined by analysis of variance (ANOVA). A response surface methodology was used to calculate the coefficients of the quadratic models proposed and to estimate the statistical significance of the regression coefficients.

Dispersive Liquid−Liquid Microextraction. Each honey sample was homogenized by manual stirring (3 min), and a representative 10 g aliquot was dissolved with acidified water (0.25 M acetic acid) to the final volume of 100 mL to obtain a 10% (w/v) solution. Diluted solution was filtered through a 0.45 μm syringe cellulose filters to remove solid particles. Three aliquots (10 mL) of diluted honey sample were subjected to the Zn reduction procedure for the determination of total PAs. In parallel three aliquots of the diluted sample were directly processed by DLLME, omitting the reduction step, for the determination of tertiary PA amounts. Under the final DLLME conditions, 5 mL portions of reduced and unreduced diluted samples were placed into 15 mL conical tubes containing 1.25 g of NaCl and the pH of the aqueous solution was adjusted at 9.6 with 5 N NaOH. Then, 500 μL of $CHCl_3$ (DLLME extractant) and 500 μL of iPrOH (DLLME disperser) was rapidly injected into the aqueous solution. The ternary component system was vigorously shaken by hand for 10 s to form a cloudy solution.

Successively, the mixture was centrifuged for 5 min at 13000 rpm (ALC centrifuge PK 120, Thermo Electron Corporation) to separate two phases. The settled phase was quantitatively transferred to a 2 mL Eppendorf vial and dried under a gentle nitrogen flow. Finally, the residue was reconstituted with 125 μL of MeOH/H_2O 2/8 v/v before the chromatographic analysis.

Zn Reduction. To determine the total PA amount, 200 mg of Zn powder was placed in a conical tube containing 10 mL of diluted honey sample (10% w/v solution in 0.25 M acetic acid). The sample solution was weakly agitated by magnetic stirring for 60 min at room temperature. Successively, the reduced sample was centrifuged (13000 rpm, 5 min) and 5 mL of supernatant was submitted to DLLME.

Strong Cation Exchange SPE. SPE was carried out as described previously by Bodi et al.6 Briefly, 3 g of honey was dissolved in 10 mL of 0.05 mM sulfuric acid solution and centrifuged at 13000 rpm for 5 min. The supernatant was passed through an SPE cartridge (Oasis MCX, 150 mg), previously preconditioned with 5 mL of MeOH and 5 mL of 0.05 mM sulfuric acid. After it was washed with 6 mL of water and 6 mL of methanol, the SPE cartridge was dried under vacuum for 10 min. Analytes

were eluted with 10 mL of 2.5% ammonia in methanol. SPE extracts were reconstituted with 750 μL of MeOH/H2O 2/8 v/v before the chromatographic analysis.
Overall recoveries of the SPE procedure were evaluated by processing one honey sample (EU multifloral honey) spiked with 2.5 μg kg−1 of each PA (n = 3). Recoveries obtained (Im, 88%; Ly, 79%; ImNO, 77%; Sn, 73%; Re, 91%; SnNO, 76%; ReNO, 97%; Em, 82%; EmNO, 94%) were comparable to those reported by Bodi et al.6 Validation of Analytical Procedure. The proposed method for determination of PAs in honey was validated with regard to extraction efficiency (EE) of DLLME, matrix effect (ME), overall recovery (R), precision, linearity, and limits of detection (LODs) and quantification (LOQs) of method.
In the case of EE, ME, and R, three sample sets were processed by optimized procedures (DLLME and Zn/DLLME), in triplicate and at three concentration levels (0.25, 2.5, and 25 μg kg−1 of honey, corresponding to 1, 10, and 100 ng mL−1). The first sample set consisted of postextraction spiked samples (matrix-matched standard), the second set consisted of pre-extraction spiked samples (spiked sample), and the last set consisted of unspiked samples (blank). Blank honey samples were not found, and the spiking experiments were performed on a honey sample (EU multifloral honey) with low contamination levels of Em, Im, and Ly. Therefore, analyte responses of unspiked sample set (blank) were subtracted to pre- and postextraction spiked samples for the determination of the analytical performance of the proposed procedure. EEs were calculated as the ratio between the analyte responses in pre- and postextraction spiked samples, whereas the Rs were defined as the ratio between the analyte responses in spiked sample and solvent solution. MEs were defined as the ratio between the slopes of calibration curves for matrix-matched and solvent-based (MeOH/H2O 2/8 v/v) standards. ME values below 80% or above 120% displayed a significant ME (signal suppression or enhance, respectively). MEs were also evaluated in five other different honey samples (Italian multifloral, acacia, citrus, sulla, and eucalyptus honeys), at a spiked level of 2.5 μg kg−1, to consider the compositional variations of this food matrix. In this case, MEs were calculated as the ratio between the analyte responses in post-extraction spiked samples and solvent solution.
Intra- and interday variations were chosen to determine the precision of the method. Intraday variability was obtained by the same set of recovery experiments, evaluating the responses of spiked samples at three levels of concentration. Interday variability was evaluated by analyzing the extracts for three consecutive days in triplicate. The precision was expressed by relative standard deviation (RSD).
The linearity of the solvent-based calibration standards was estimated in the working range of 0.2−100 ng mL−1 (corresponding to the range 0.05−25 μg kg−1), with 10 calibration levels, each injected in triplicate. The regression curves were tested with the analysis of variance (ANOVA), and a linear model was found to be appropriate over the tested concentration range ($R2 \geq 0.999$).
LODs and LOQs were estimated via a calibration approach,21 using artificial honey as a pseudoblank. For this purpose, five concentration levels close to the expected LOD, including the zero spiking level (0, 0.025, 0.05, 0.075, and 0.1 μg kg−1), were prepared in duplicate and analyzed by the entire analytical procedure. Then, the estimated limits for Im, Ly, Sn, and Re were verified experimentally by spiking a honey sample with undetectable levels of these analytes (sample H23, Table 2S in the Supporting Information), with PAs at the level 0.05 μg kg−1.
Statistical Analyses. Data were expressed as mean ± standard deviation of triplicates. The data were statistically analyzed using Statgraphic Centurion XVI software, version 16.1. Statistically significant differences were evaluated by a multiple sample comparison procedure (ANOVA and Tukey HSD multiple comparison tests).

■ RESULTS AND DISCUSSION

UHPLC-MS/MS Analysis. The determination of PAs was obtained by UHPLC-MS/MS. Several columns, organic mobile phases, and modifiers were evaluated to achieve the best chromatographic performance (reduction of peak tailing and better resolution) and the most intense ionization of the analytes. Particularly, LC conditions were optimized to reduce matrix effects and to achieve a

satisfactory separation of Im and Ly, two PA isomers that tend to coelute. The best LC separation and MS ionizations of the analytes were obtained with the conditions described in UHPLC-MS/MS Analyses, exhibiting an efficient separation of the analytes in a total run time of 13 min.

The multiple reaction monitoring (MRM) mode was selected as a quantitative and confirmatory method to enhance the selectivity and sensitivity of targeted analysis. MS/MS spectra of [M + H]+ ions were studied to identify MRM transitions to be monitored for each analyte. Product ions at m/z 94, 120, and 138, related to the necine-core structure of 1,2-unsaturated PA, and at m/z 118 and 136, typical of PANOs,22,23 were selected for the majority of analytes. ESI and MS/MS parameters were carefully evaluated to maximize the analyte responses. Quantification of the target analytes was performed adding three MRM transitions (two for Em). In addition, the ratio between the intensities of MRM transitions was used to confirm the identity of the analytes and to fulfill the 2002/657/EC regulation.

Optimization of DLLME. Considering the low levels in food products and the complexity of food matrices, the trace analysis of PAs requires a sample treatment before instrumental analysis in order to concentrate the analytes, to remove interfering matrix components, and to provide a highly sensitive and selective analytical procedure. In this study, DLLME was evaluated as an extraction and cleanup technique to obtain a rapid and easy multiresidue method for the analysis of PAs in honey. Sugars and proteins are the main honey interferences to remove with the sample preparation procedure, and DLLME proved to be a suitable technique to eliminate these honey constituents and to extract the minor components.20 In addition, the solubility of honey in water facilitates the sample treatment step, since its diluted solution can be directly used as the aqueous phase in DLLME.17 In fact, it was successfully employed in the determination of pesticides, antibiotics, and phenolic compounds in honey.17,18,20,24 Parameters affecting DLLME efficiency (pH and ionic strength of the sample solution, type and volume of extraction, and disperser solvents)17−19 were systematically investigated and optimized to achieve the best PA extraction efficiency.

Optimization experiments were performed in triplicate using diluted artificial honey (10 mL of 10% w/v aqueous solution, corresponding to 1 g of honey) spiked with 25 μg kg−1 of each PA and PANO. DLLME extracts were reconstituted with 250 μL of MeOH/water 2/8 v/v before UHPLC-MS/MS analysis.

Effect of pH on DLLME Efficiency. Initially, the influence of pH on DLLME efficiency was investigated, because pH might strongly affect the LLE efficiency of ionizable compounds, such as PAs.17−20,25,26 Four pH values (2, 7, 9.6, and 11.2) were investigated using 1000 μL of $CHCl_3$/MeCN 4/6 v/v as an extractant/disperser mixture. At pH 2, no analyte was recovered by DLLME. However, on an increase in the pH, some PAs were extracted by DLLME, and acceptable EE values for Em (45 ± 3), Re (28 ± 2), and Sn (63 ± 5) were obtained at pH 9.6. PAs are bases (pKa from 10.6 to 12.4)27 that at acid pH form salts that are very soluble in water. On the other hand, the free bases are usually not soluble in water and soluble in organic solvents (chloroform or diethyl ether).28 Therefore, pH affects the PA solubility in LLE and the alkaline pH enhances DLLME efficiency. Therefore, a pH of 9.6 was selected as the optimal condition. At this pH value, a low amount of analytes was ionized (0.2−9% protonated form), which in turn increased the extraction efficiency.19 Nevertheless, PANOs were not extracted under alkaline conditions, and insufficient EEs (<1%) were observed for the two monoester PAs, Im and Ly.

Effect of Ionic Strength on DLLME Efficiency. As is the case for pH, salt addition has a significant effect on the extraction efficiency of hydrophilic compounds, because the salting out effect reduces their water solubility.17−20 In this regard, the influence of ionic strength was evaluated by testing NaCl and $(NH_4)_2SO_4$, in order to improve the extraction of the analytes presenting lower recoveries. The results indicated that the salt addition (2 g per 10 mL) to the diluted artificial honey (pH 9.6) strongly improved DLLME efficiency (Figure S2 in the Supporting Information). Particularly, both salts provided an exhaustive extraction of Em, Re, and Sn (EEs 71−101%) and significantly increased the EEs of Im (6−9%) and Ly (EEs 5−7%). NaCl showed better EEs than did $(NH_4)_2SO_4$, for these two PAs ($p < 0.05$), and thus it was chosen for the next experiments. However, according to these

results, the DLLME efficiency was still very low for PANOs (EEs < 3%), Im, and Ly. The observed poor DLLME efficiency is related to the higher hydrophilic character of these compounds (log P <−0.5) in comparison to the rest of the analytes (log P > 0.6).27 Effect of Extractant and Disperser Solvents on DLLME Efficiency. Further, taking into account the parameters that mainly influence DLLME efficiency,17−19 the types of extractant and disperser solvents were evaluated. Among the tested solvents with densities higher than that of water, $CHCl_3$ showed better EEs for PAs and it was chosen as the DLLME extractant (data not shown). For the selection of the disperser solvent, five solvents (MeCN, THF, MeOH, EtOH, and iPrOH) with the required properties for a DLLME disperser were investigated. The experimental conditions used were pH 9.6, NaCl 20% w/v, $CHCl_3$ 400 μL, and disperser volume 600 μL, and the results are illustrated in Figure 1. EtOH and iPrOH exhibited the best EEs for all analytes. In particular, iPrOH showed a strong influence on the extraction efficiency of the most hydrophilic analytes. It significantly improved ($p < 0.05$) the EEs of monoester PAs Im (35% ± 0.2) and Ly (32% ± 0.3) and also allowed extraction of SnNO and EmNO by DLLME with EEs of 21 and 15%, respectively (Figure 1). On the basis of these results, iPrOH was chosen as the disperser in DLLME.

Optimization of DLLME Parameters by Response Surface Design. Once the DLLME solvents, pH, and ionic strength of the aqueous solution were selected, the extractant and disperser volumes and salt amount were investigated in order to enhance the efficiency of DLLME toward the most hydrophilic analytes. The interactions among the experimental variables should be considered during the optimization of the extraction conditions, because they can directly or indirectly affect the process, by mutually interacting. Hence, a response surface design was applied to simultaneously assess the effect of salt amount (A), extractant volume (B), and disperser volume (C) on DLLME efficiency. In detail, a Box−Behnken design (BBD) 2-factor interaction design was employed to study and optimize three experimental factors (A, 15−25% w/ v NaCl; B, 400−1000 μL $CHCl_3$; C, 500−1000 μL iPrOH). EEs of PAs and PANOs were considered as response variables to maximize the extraction yields. For ImNO and ReNO no adequate EE values were observed under the investigated conditions; thus, these analytes were excluded from chemometric analysis.

The statistical significance of the experimental factors A−C and their first-order interactions were determined by analysis of variance (ANOVA), and the estimated standardized effects on response variables are shown in Pareto charts (Figure 2).

All the three experimental factors significantly affected the DLLME efficiency of the most hydrophilic analytes, Im, Ly,SnNO, and EmNO ($p < 0.05$). The disperser volume (C) and NaCl amount (A) showed a significant effect on the EEs of Sn and Re, respectively, whereas no significant main effects of A−C ($p > 0.05$) on the EE of Em were observed (Figure 2).

Factors A and C strongly affected DLLME efficiency (Figure 2), and their influence is clearly indicated in Figure 3, which reports the response surface plots for the combined effect of experimental factors, after excluding nonsignificant terms ($p > 0.05$). For Im, Ly, SnNO, and EmNO, an evident increase in EE was achieved by increasing the NaCl amount (A) and disperser volume (C) (Figure 3). These results pointed out that these two factors highly affected DLLME efficiency in the case of the most hydrophilic analytes studied. According to literature data, high recoveries in DLLME are reached on increasing the ionic strength of the aqueous phase and, consequently, decreasing the water solubility of analytes.17−20 With regard to the disperser volume, it generally affects the extraction efficiency by promoting either the formation of the cloudy solution or the degree of the dispersion of extraction solvent in the aqueous phase.17−19 In the present study, the good results observed using iPrOH as disperser solvent are probably due also to its coextraction action. The use of iPrOH as disperser solvent produced an increase in the settled phase volume, in comparison with the extractant volume injected into the sample; hence, the improvement of DLLME efficiency can be attributed to the extraction of a portion of the analytes into iPrOH along with its partitioning into the extractant phase. Similar observations were also reported previously for polar compounds extracted by DLLME.29 In the case of Sn effect on EE (Figure 3), since their exhaustive extraction was already achieved by optimizing the conditions of the aqueous phase during the preliminary experiments.

Finally, a multiple response analysis was performed to simultaneously consider the effects of investigated DLLME factors on the EEs of Im, Ly, Sn, Re, SnNO, and EmNO and to maximize the extraction efficiency. The optimal DLLME conditions extrapolated by the experimental design were 25% w/v NaCl, 1000 μL of CH_3Cl, and 1000 μL of iPrOH (desirability of 87%). These most favorable conditions were experimentally corroborated, and the results are reported in Figure 1. Optimal DLLME conditions provided good EEs for Im, Ly, Sn, Re, and Em (73−104%), whereas lower EE values were obtained for SnNO and EmNO (51 and 54%, respectively).

In order to further reduce the quantities of organic solvents, half of the optimized volumes (5 mL of 10% (w/v) honey solution containing 25% w/v of NaCl, 500 μL of CH_3Cl and iPrOH) was tested and no significant difference was observed.

Therefore, DLLME conditions reported in Dispersive Liquid−Liquid Microextraction were used for the following studies.

Zn Reduction. As shown in Figure 1, the optimized DLLME conditions allowed us to improve greatly the extraction efficiency toward PAs (Im, Ly, Re, Sn, and Em).

Nevertheless, low EE values for EmNO and SnNO and insufficient recoveries for ImNO and ReNO were obtained using DLLME as the extraction technique. These results suggest that DLLME is not a suitable procedure for the effective extraction of PANOs due to their high water solubility. Nevertheless, since PANOs can be partially reduced to the corresponding tertiary amines in the gut, resulting in equal toxicity,1,30 it is mandatory to include PANOs in the analytical methods for the safety assessment of PA-containing products.

To overcome the limitation of DLLME and to extend the applicability of the method to PANOs, reduction with Zn powder in acidic aqueous solution was employed to convert PANOs in the corresponding tertiary bases.1,2,31 Therefore, to gain information on PA and PANOs level in honey, for each sample two aliquots, reduced and unreduced aqueous solutions, were processed by DLLME and analyzed by UHPLC-MS/MS. The difference between total PA (reduced aliquot) and tertiary PA (unreduced aliquot) amounts reflects the amount of PANOs originally present in the sample.

Preliminarily, to establish the reduction conditions (Zn amount, acid conditions, and time reaction) and to verify the chemical stability of PAs and PANOs and the completeness of the reaction, several experiments were carried out. In these experiments, an 10% (w/v) aqueous solution of artificial honey fortified with PAs at the 100 μg kg^{-1} level, was used as test matrix. Under conditions described in Zn Reduction, the complete reduction of ImNO, ReNO, SnNO, and EmNO (conversion PANOs > 98%) was observed without significant degradation of Em, Im, Ly, Re, and Sn (Figure 4).

Matrix Effects. The matrix effect (suppression or enhancement of instrumental response due to coeluting matrix constituents) is an important issue of LC-MS quantitative methods because it primarily impairs the accuracy of the quantitative measurements. In addition, the type and concentration of matrix interferents might change between different samples of the same matrix, thus creating a negative influence on the precision of the quantitative LC-MS method.

Therefore, in the validation of new analytical procedures, a careful evaluation of ME must be performed to establish the most suitable quantification method (standard or matrix matched calibration curves).

MEs of the developed DLLME procedure were investigated by comparing the slopes of matrix-matched and standard solution calibration curves (0.25−25 μg kg^{-1} corresponding to 1−100 ng mL^{-1}) in both reduced and unreduced DLLME extracts. MEs observed for all analytes (Table 1) were negligible in both procedures (94−105% for DLLME extracts and 81−96% for Zn-DLLME extracts), proving that DLLME is able to remove and/or reduce honey interferents during the extraction and cleanup of analytes.

Afterward, MEs were evaluated in honey samples of different botanical origins (Italian multifloral, acacia, citrus, sulla, EU multifloral, and eucalyptus honeys) to assess the influence of compositional variations (sugars, proteins, and minor compounds) and the reproducibility of the overall procedure. No statistically significant difference (Im, $p = 0.88$; Ly, $p = 0.09$; Sn, $p = 0.12$; Re, $p = 0.09$; Em, $p =$

0.10) was observed among the different honeys analyzed, further supporting the good accuracy and repeatability of the proposed sample preparation.

Globally, the results obtained during ME assessment confirmed the suitability of solvent calibration curves for the accurate quantification of PAs in honey, thus avoiding the use of matrix-matched calibration curves.

Analytical Performance. The optimized analytical procedures (DLLME and Zn-DLLME) for analysis of PAs in honey were validated with regard to selectivity, linearity, accuracy (in terms of extraction efficiency, overall recovery, and comparison with SPE procedure),6 precision, and sensitivity.

The LC-MS/MS method proposed was highly selective due to data acquisition by the MRM mode with three characteristic transition precursor/product ions, except for Em. Moreover, MRM ratios (Table S3 in the Supporting Information) were used as additional identification criteria (tolerance of less than 20% of the expected ratio), thus fullfilling EU guidelines (Commission Decision 2002/657/EC) with 5.5 identification points for the confirmation of analytes with LC-MS/MS detection.

With regard to linearity, given that no significant MEs were observed, solvent-based calibration standards were proposed for PA quantification. MS responses of all PAs were found to be linear over a wide concentration range (0.2−100 ng mL−1, corresponding to 0.05−25 μg kg−1 of honey) with a correlation coefficient greater than 0.999 for a linear model of the Information).

The accuracy of the proposed DLLME protocols was investigated in honey samples spiked at three different PA levels (0.25, 2.5, and 25 μg kg−1). Extraction efficiencies (EEs) of both sample preparation procedures (DLLME and Zn-DLLME) varied from 68 (Im) to 106% (Em) in DLLME and from 69 (Ly) to 99% (Em) in Zn-DLLME (Table 1), and no significant differences were observed between the two procedures ($p > 0.05$) and spiking levels ($p > 0.05$). Moreover, EEs of Im, Ly, and Em in honey were in agreement ($p > 0.05$) with the data shown for artificial honey under optimal conditions (Figure 1). In the case of Sn and Re, instead, slightly lower values were observed in the real matrix ($p < 0.05$). The overall recoveries (Rs) of DLLME and Zn-DLLME at three different levels, established with solvent-based calibration curves, are also given in Table 1. R values (DLLME, 71−102%; Zn-DLLME, 63−103%) indicate an exhaustive extraction and accurate determination of five Pas from honey. Finally, for the assessment of accuracy, three naturally contaminated honey samples (samples H1, H16, and H21, Table S2 in the Supporting Information) were processed by DLLME and SPE6 protocols to compare the qualitative/quantitative results. Figure 5a reports the MRM chromatograms of PAs found in real honey samples. Both methods allowed detection of Im, Ly, Sn, and Em in the samples, and the PA levels showed good agreement between DLLME and SPE (Table 2).

The precision of the method, evaluated as intra- and interday variations (RSD) of five PAs in spiked samples at three concentration levels, was less than 12% (Table 1), indicating that satisfactory precision was achieved.

Honey samples containing no detectable analytes were not found; therefore, an evaluation of method sensitivity using blank samples spiked at very low concentration levels was not possible. LODs and LOQs were calculated by spiking artificial honey (pseudoblank) at levels close to the expected LOD (0−0.1 μg kg−1, five levels in duplicate), according to guidance documents.21,32 The estimated LOQs varied between 0.03 and 0.06 μg kg−1 (Table 1), and they are lower than those reported for other sample preparation techniques (SPE and QuECh-ERS).6,14 Later, the limits estimated for Im, Ly, Sn, and Re were corroborated by spiking a honey sample free of these analytes (sample H23) with PA concentrations close to the LOQ (0.05 μg kg−1). Chromatograms of spiked and unspiked sample H23 reported in Figure 5b provide the proof of the detection capabilities of the method.

Analysis of Commercial Honey Samples. The optimized procedures for determination of tertiary PAs (DLLME) and total PAs (Zn-DLLME) were subsequently applied to 25 honey samples from Italian retail markets (Table 2). The results showed that all analyzed samples contained at least one of the PAs, and the overall contamination levels ranged from 0.1 to 16.1 μg kg−1 for tertiary PAs and from 0.2 to 17.5 μg kg−1 for total PAs. Em was the most abundant PA, detected in all samples with

concentrations ranging from 0.1 to 15.6 μg kg−1, followed by Im and Ly (detected as tertiary PA in 80 and 72% of samples, respectively, and as total PA in all analyzed retail honeys from Europe.1,5,6 The higher levels of total PAs in comparison to the tertiary PAs (Table 2), indicated the presence of significant PANO amounts in the analyzed samples. In this regard, the method here proposed is suitable for easy and correct determination of PAs and PANOs.

In summary, a simple and inexpensive extraction procedure, based on DLLME, was proposed for the determination of Pas of food relevance, and their corresponding N-oxide derivatives (PANOs), in honeys.

DLLME conditions were investigated mainly to enhance the efficiency of DLLME toward the most hydrophilic analytes (Im, Ly, and PANOs), and the main parameters affecting their extraction efficiency were pH, ionic strength, and type and volume of disperser solvent. However, optimal DLLME conditions provide an exhaustive extraction (EEs 73−104%) only for tertiary PAs. Thus, to extend the applicability of the method to PANOs, a reduction step before DLLME was employed to gain the total PA levels.

The two sample preparation procedures (DLLME and Zn-DLLME) were validated, and the results revealed that DLLME combined with Zn reduction rendered quantitative recoveries for the determination of tertiary PAs and total PAs in honey, with moderate solvent consumption and insignificant matrix effects during ESI (+) ionization. Consequently, the targeted analytes could be quantified by comparison against pure standard solutions, avoiding the use of matrix-matched calibration for quantification as recommended in previous protocols.6,14 UHPLC-MS/MS analysis improves the selectivity of the method, and the attained LOQs are low enough to monitor the PA levels in real samples.

Although the accuracy of the proposed procedure has been demonstrated to be equivalent to the most widely used method in the analysis of PAs in honey (strong cation exchange SPE), DLLME is less costly and requires a reduced volume of organic solvents for sample preparation with respect to other procedures.6,14 Finally, the suitability of the proposed method for PA analysis to other matrices was evaluated by processing infusions, green and black tea samples. Preliminary results (data not shown) suggested extraction efficiency comparable to that obtained with honey, but significant matrix effects (<80%) for most analytes, depending on the tested herbal matrices, were observed. These data emphasize the need to carefully evaluate the matrix effects for each food matrix in order to achieve accurate quantitative methods for PA analysis in food products. Further studies are in progress to develop strategies that allow extension of the proposed method to tea and infusions.

Notes

The authors declare no competing financial interest.

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Table 1. Analytical Performance of DLLME and Zn-DLLME Methods for Determination of Tertiary PAs (DLLME) and Total PAs (Zn-DLLME)

	Sensitivity ($\mu g\ kg^{-1}$)				
	Im	Ly	Sn	Re	Em
MDL ($\mu g\ kg^{-1}$)	0.01	0.01	0.01	0.02	0.01
MQLs ($\mu g\ kg^{-1}$)	0.04	0.03	0.04	0.06	0.04
	Matrix Effect (ME)				
	Im	Ly	Sn	Re	Em
DLLME	103.5	105.0	102.9	93.7	96.9
Zn/DLLME	86.8	95.5	82.1	88.4	81.4
	Extraction Efficiency (EE) ± SD(n = 3)				
spiking level	Im	Ly	Sn	Re	Em
DLLME (0.25 $\mu g\ kg^{-1}$)	68.2 ± 3.3	78.7 ± 2.6	76.2 ± 1.3	79.3 ± 7.2	102.2 ± 5.8
DLLME (2.5 $\mu g\ kg^{-1}$)	70.5 ± 1.9	70.3 ± 2.6	85.6 ± 3.0	86.8 ± 4.8	105.7 ± 10.7
DLLME (25 $\mu g\ kg^{-1}$)	71.4 ± 4.4	72.1 ± 6.4	86.1 ± 3.6	90.0 ± 6.6	89.6 ± 3.2
Zn/DLLME (0.25 $\mu g\ kg^{-1}$)	70.3 ± 5.2	78.5 ± 3.0	83.4 ± 2.9	81.1 ± 6.7	99.3 ± 2.0
Zn/DLLME (2.5 $\mu g\ kg^{-1}$)	72.3 ± 9.0	69.1 ± 4.1	83.1 ± 0.8	76.5 ± 7.2	95.2 ± 10.6
Zn/DLLME (25 $\mu g\ kg^{-1}$)	73.4 ± 5.2	72.7 ± 5.3	80.0 ± 4.2	79.1 ± 3.0	97.6 ± 7.2
	Recovery (R) ± SD(n = 3)				
spiking level	Im	Ly	Sn	Re	Em
DLLME (0.25 $\mu g\ kg^{-1}$)	70.6 ± 3.4	83.4 ± 2.8	78.4 ± 1.4	74.2 ± 6.8	102.2 ± 5.8
DLLME (2.5 $\mu g\ kg^{-1}$)	73.0 ± 2.0	74.4 ± 2.8	88.1 ± 3.0	81.3 ± 4.5	102.5 ± 10.4
DLLME (25 $\mu g\ kg^{-1}$)	73.9 ± 4.5	76.4 ± 6.8	88.6 ± 3.8	84.3 ± 6.2	86.8 ± 3.1
Zn/DLLME (0.25 $\mu g\ kg^{-1}$)	67.4 ± 5.0	71.7 ± 2.8	91.8 ± 3.2	81.0 ± 6.7	102.6 ± 2.1
Zn/DLLME (2.5 $\mu g\ kg^{-1}$)	63.3 ± 7.9	60.5 ± 3.6	76.9 ± 0.7	69.1 ± 6.5	78.5 ± 8.8
Zn/DLLME (25 $\mu g\ kg^{-1}$)	63.7 ± 4.5	69.4 ± 5.1	65.6 ± 3.5	69.9 ± 2.6	79.5 ± 5.9
	Intra-/Interday Precision (RDS) (n = 3)				
spiking level	Im	Ly	Sn	Re	Em
DLLME (0.25 $\mu g\ kg^{-1}$)	5/10	3/6	2/5	9/11	6/9
DLLME (2.5 $\mu g\ kg^{-1}$)	3/6	4/7	3/4	6/9	10/9
DLLME (25 $\mu g\ kg^{-1}$)	6/8	9/10	4/5	7/9	4/5
Zn/DLLME (0.25 $\mu g\ kg^{-1}$)	7/10	4/7	3/7	8/11	2/8
Zn/DLLME (2.5 $\mu g\ kg^{-1}$)	12/10	6/8	1/5	9/10	11/11
Zn/DLLME (25 $\mu g\ kg^{-1}$)	7/8	7/7	5/6	4/6	7/8

Table 2. PA Levels in Retail Honey Samples Processed by the Proposed Methods (DLLME and Zn-DLLME) and SPE for Selected Naturally Contaminated Honeys (H1, H16, and H21)[a]

	PA level ($\mu g\ kg^{-1}$ ± standard deviation)[b]				
	Im	Ly	Sn	Re	Em
H1 by DLLME	0.45 ± 0.03	0.08 ± 0.01	<MDL	<MDL	15.62 ± 1.10
H1 by SPE	0.45 ± 0.03	0.09 ± 0.01	<MDL	<MDL	15.90 ± 1.14
H16 by DLLME	0.12 ± 0.01	0.09 ± 0.01	0.15 ± 0.01	<MDL	3.54 ± 0.19
H16 by SPE	0.14 ± 0.01	0.07 ± 0.01	0.16 ± 0.02	<MDL	3.64 ± 0.26
H21 by DLLME	0.67 ± 0.06	0.58 ± 0.02	<MDL	<MDL	8.10 ± 0.46
H21 by SPE	0.64 ± 0.05	0.59 ± 0.03	<MDL	<MDL	8.60 ± 0.53
	tertiary PA in real sample (n = 25)[c]				
	Im	Ly	Sn	Re	Em
% > MDL	80	72	68	24	100
minimum ($\mu g\ kg^{-1}$)	<MQL	<MQL	<MQL	<MQL	0.10
maximum ($\mu g\ kg^{-1}$)	0.67	0.58	0.16	0.40	15.62
mean ($\mu g\ kg^{-1}$)	0.20	0.12	0.10	0.22	1.98
	total PA in real sample (n = 25)[d]				
	Im	Ly	Sn	Re	Em
% > MDL	100	100	76	32	100
minimum ($\mu g\ kg^{-1}$)	<MQL	<MQL	<MQL	<MQL	0.16
maximum ($\mu g\ kg^{-1}$)	1.14	0.88	0.16	0.53	16.80
mean ($\mu g\ kg^{-1}$)	0.33	0.17	0.10	0.19	2.32

[a]Data were corrected by overall recoveries. [b]n = 3. [c]Unreduced samples (DLLME). [d]Reduced samples (Zn-DLLME).

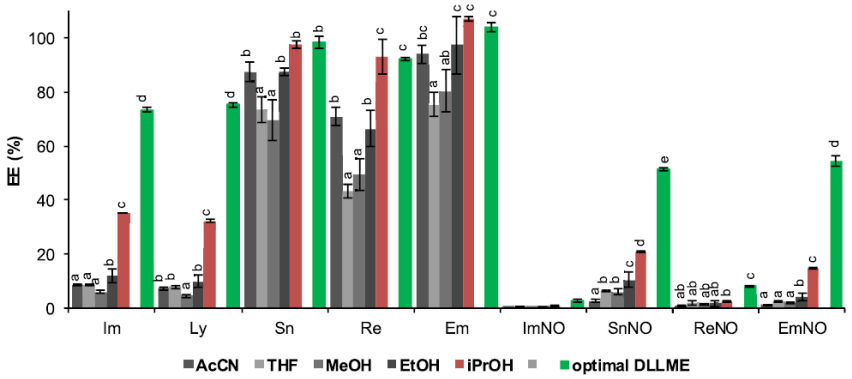

Figure 1. Effect of different disperser solvents on DLLME efficiency. Experimental conditions: n = 3; 10 mL of 10% w/v sugar aqueous solution; spiked at 25 μg kg^{-1} of each PA and PANO; pH 9.6; NaCl 2 g; $CHCl_3$ 400 μL; disperser volume 600 μL. Different superscript letters within each compound indicate significant differences among experimental conditions ($p < 0.05$).

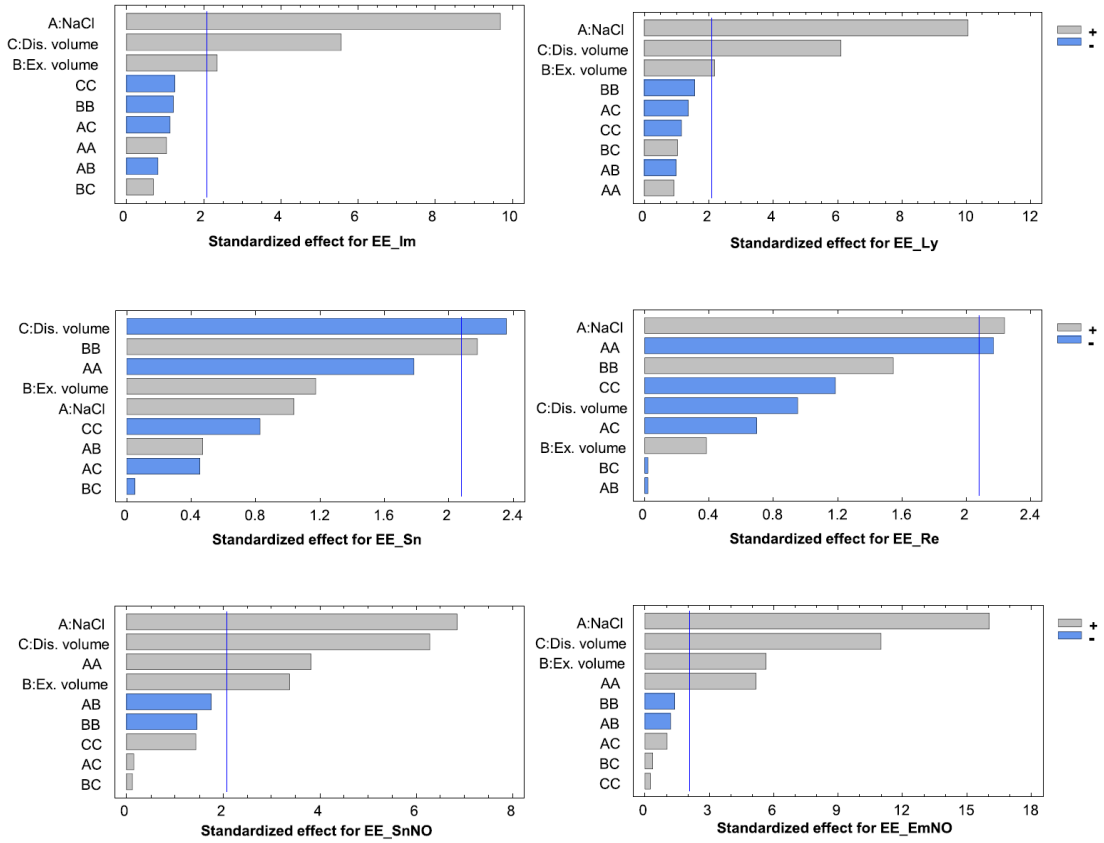

Figure 2. Standardized effect Pareto charts of Im, Ly, Sn, Re, SnNO, and EmNO for the response surface design. Differences in the bar shadings indicate positive and negative effects of the experimental factors on the response variables a–f, and the vertical line corresponds to statistical significance at the 95% confidence level.

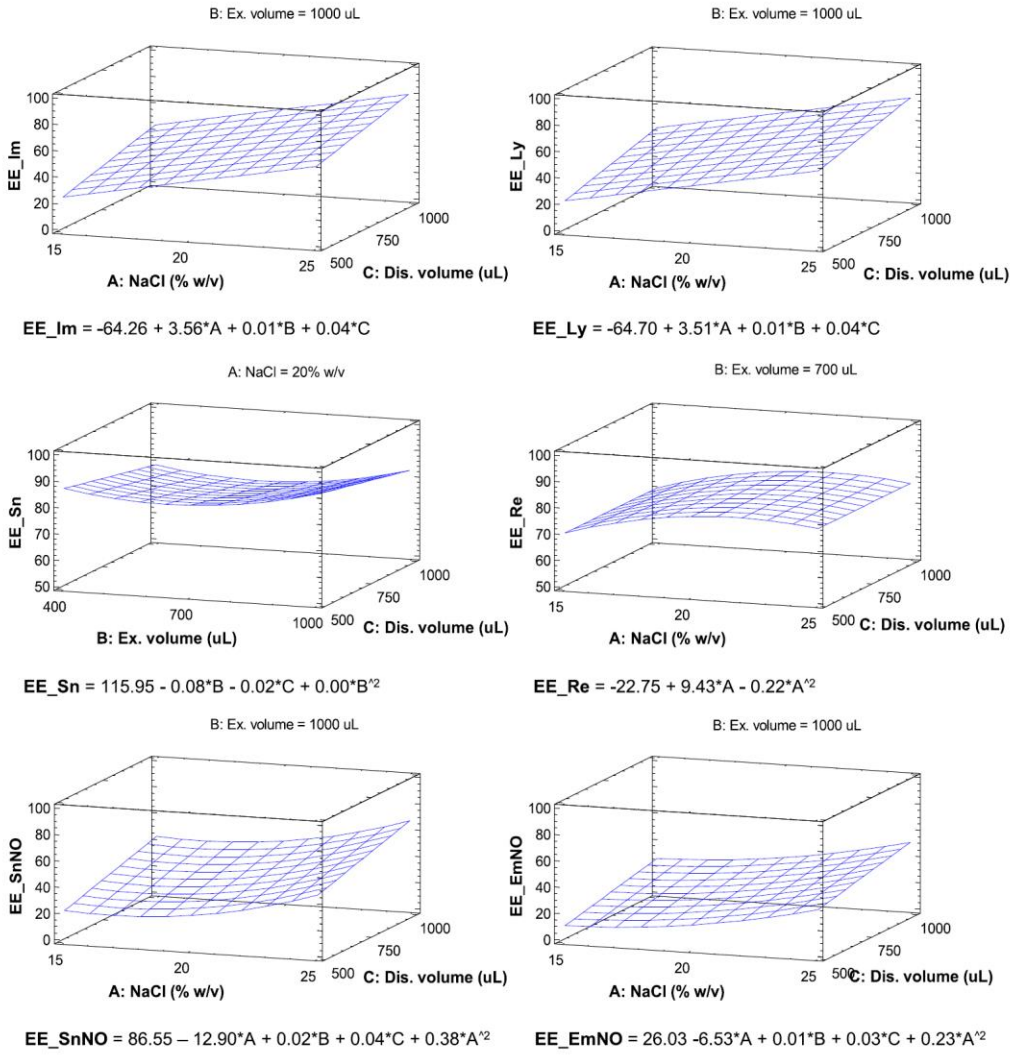

Figure 3. Response surface plots and the equations of the fitted models for Im, Ly, Sn, Re, SnNO, and EmNO, after excluding nonsignificant ($p > 0.05$) experimental factors (Figure 2).

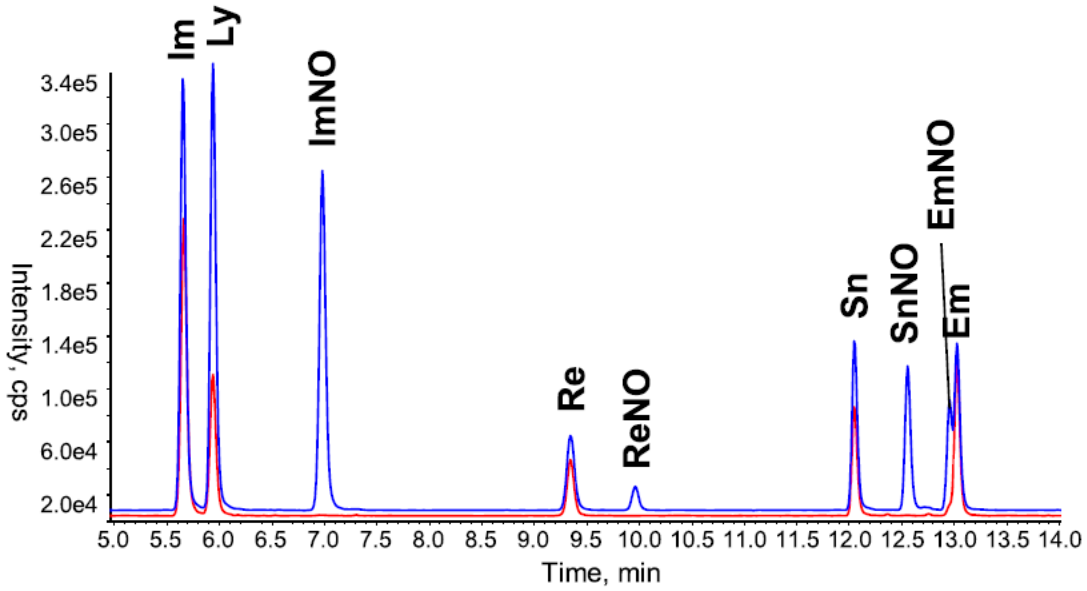

Figure 4. UHPLC-MRM chromatograms of PA and PANO mixture before (blue line) and after (red line) Zn reduction under the optimal conditions (artificial honey fortified with PAs at 100 μg kg^{-1} level).

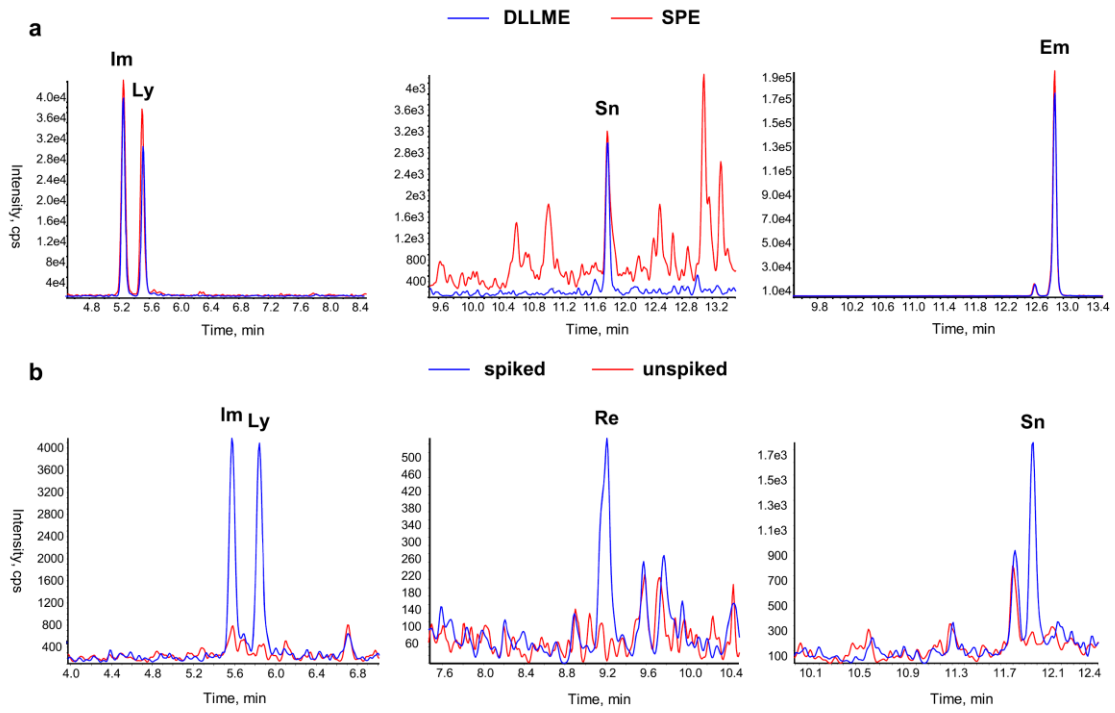

Figure 5. UHPLC-MRM chromatograms of (a) naturally contaminated honey samples processed by DLLME (blue lines) and SPE (red lines) and (b) unspiked (red lines) and spiked (blue lines) honey sample (Im, Ly, Sn, and Re levels close to the LOQs, 0.05 μg kg^{-1}) processed by DLLME.